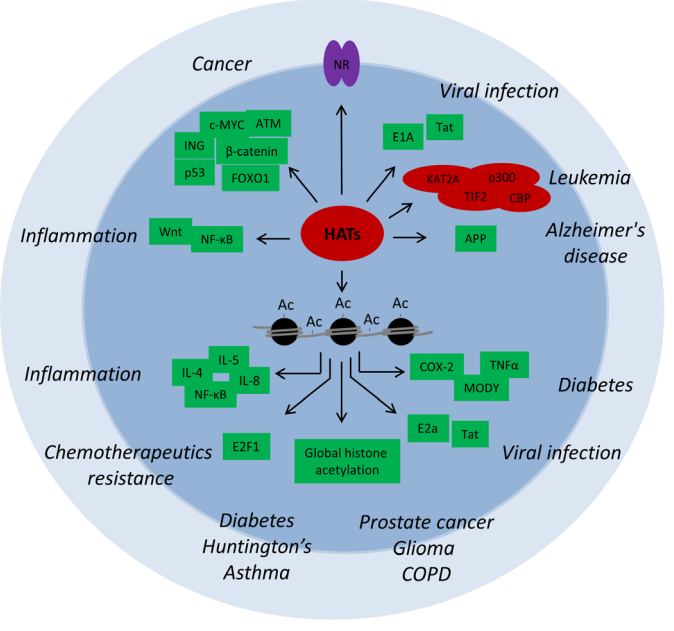
Kinetics and inhibition of enzymes in early stage drug discovery

Wapenaar, Hannah

Chapter 2

Experimental approaches toward histone acetyltransferase inhibitors as therapeutics



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The “histone code”

Post-translational modifications (PTM) of chromatin-associated proteins (e.g. histones) play an important role in the epigenetic regulation of gene transcription. These modifications are crucial for the ‘’fine-tuning’’ of cellular responses and increase the adaptability of cells to a changing environment. Histone proteins are highly conserved in eukaryotic species and function as building blocks to package the DNA into folded nucleosomal units that form chromatin fibers. The nucleosome contains 146 base pairs of DNA wrapped around a histone octamer core, which structure has been described as beads on a string (1,2). Core histones are globular except for their N-terminal tails, which protrude from the DNA-histone complex and are exposed to the environment. The histone tails are therefore accessible for modification by enzymes. PTMs of histone tails include acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation (Figure 1)(3,4). These modifications affect the chromatin structure, which has an influence on the accessibility of the DNA to transcription factors. Additionally, it is thought that they form a ‘histone code’ on the N-terminal histone tails, that are ‘read’ by signaling proteins or complexes, which in turn activate signaling pathways (5). Histones have therefore an important function in epigenetic regulation of gene transcription and could additionally play a role in in nuclear signaling complexes.

Histone acetylation

Acetylation is one of the main PTMs that form the histone code. Acetylation of lysine residues on the histone tails is directly involved in regulation of gene transcription. Already in 1964, it was shown that histone binding to DNA inhibited gene transcription (6). In addition, it was shown that chemical acetylation of histones could reduce this inhibiting effect. The reduced inhibitory effect of histone acetylation on gene transcription can be explained by a reduced packing of the chromatin structure. This is due to reduction of the attractive forces of the positively charged histone tails and the negatively charged

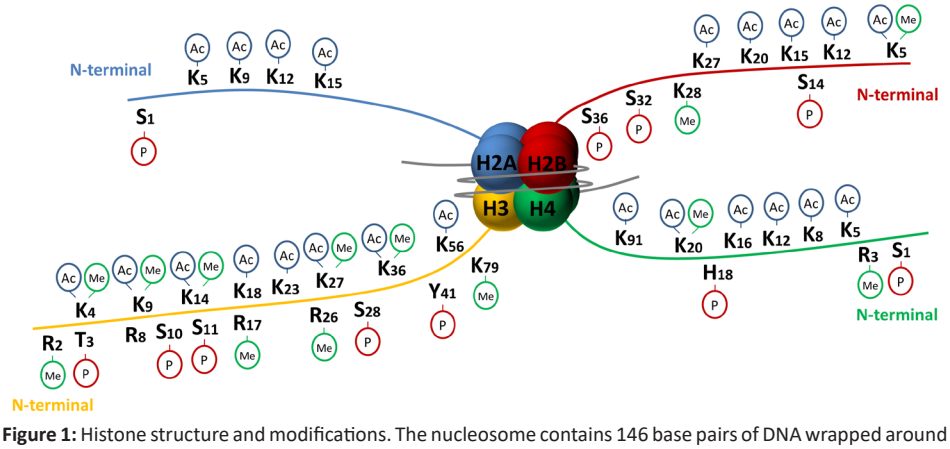

Figure 1: Histone structure and modifications. The nucleosome contains 146 base pairs of DNA wrapped around a histone octamer core, which consists of two copies of histone H2A, H2B, H3 and H4. Histone N-terminal tails, which protrude from the DNA-histone core complex and are exposed to the environment and are therefore accessible for modification by enzymes. The most common post-translational modifications of N-terminal histone tails include acetylation (Ac), phosphorylation (P) and methylation (Me).

DNA. The reduced packing of the chromatin structure allows transcription factors to bind to the DNA, thus promoting gene transcription. Indirectly, histone acetylation can regulate gene transcription through interactions with bromodomains. Bromodomains are protein interaction domains that recognize ε-N-lysine acetylation and are often part of multidomain proteins. Proteins containing bromodomains are mostly transcription factors and transcriptional regulators (e.g. the BRD and BAZ family), possibly using acetylated lysine tails as anchor to get in close proximity to the DNA. Additionally, several histone modulators (histone methyl- and acetyltransferases) contain bromodomains, suggesting that acetylation of the histone tail lysines can precede subsequent modification of the 'histone code' (7).

Acetylation levels are regulated by a dynamic equilibrium between acetylation and deacetylation reactions. Histone acetyltransferases (HATs) are enzymes that catalyze lysine acetylation reactions. Histone deacetylases (HDACs) catalyze the hydrolysis of acetylated lysine residues. Dysregulation of the acetylation-deacetylation equilibrium is implicated in disease, such as for example cancer or cardiac diseases. This chapter will focus on the current knowledge on different families of HATs, their connection to specific diseases and their respective small molecule inhibitors.

Histone acetyltransferases (HATs)

HATs acetylate histones by transferring the acetyl group from acetyl coenzyme A to the ε-amino groups of lysine residues on the histone tails. HATs not only acetylate histones, but several non-histone proteins as well. There are several families of HATs consisting of more than 20 subtypes currently discovered in plants, animals and fungi. The main families are GNAT (Gcn5 (general control of amino acid synthesis protein 5) -related N-acetyltransferases), p300/CBP (CREB (cAMP response element binding protein) -binding protein) and MYST (MOZ, YBF2/SAS3, SAS2 and TIP60) with additionally the TAFII250 (TATA-binding protein (TBP)-associated factor 250) and nuclear receptor coactivator families. This chapter will focus on the human homologs, which are classified as KAT (lysine (K) acetyltransferases) 1, 2 and 5 – 8 in addition to p300 and CBP. They are, except for KAT1, divided in the three families: GNAT (KAT2A and B), MYST (KAT5, 6A and 6B, 7, 8) and p300/CBP (p300 and CBP) based on their sequence homology. Figure 2A shows the evolutionary relationship between the human HATs and their families.

HATs from all families share a structurally conserved central catalytic domain that mediates interactions with the cofactor, acetyl coenzyme A, but have divergent N and C-terminal domains (8). The central core domain plays a role in the catalysis of the acetylation that is shared between HATs, the N and C-terminal domains contain specific domains that recruit the HAT to the correct location in the genome (Figure 2B). The p300/CBP and GNAT family HATs contain a bromodomain that can facilitate interactions with modified histones. P300/CBP family HATs contain a kinase-inducible (KIX) domain that can interact with kinases, resulting in phosphorylation of the HAT (9,10). MYST and p300/CBP family HATs contain a cysteine-rich, zinc-binding domain, which could facilitate binding directly to DNA. Some MYST family HATs contain in addition an N-terminal chromodomain, which binds to methylated lysine residues.

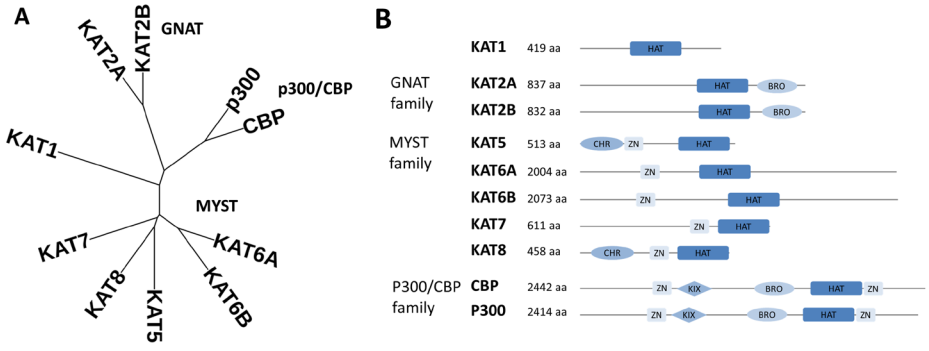

Figure 2: A) Phylogenetic tree of human HAT enzymes showing the evolutionary relationship between HATs and their families. GNAT = Gcn5-related N-acetyltransferases, MYST = MOZ, YBF2/SAS3, SAS2 and TIP60, CBP = CBP (cAMP response element binding protein) binding protein. B) HAT subtypes and their domains. The p300/CBP and GNAT family HATs contain a bromodomain that can facilitate interactions with modified histones. P300/CPB family HATs contain a kinase-inducible domain that can interact with kinases, resulting in phosphorylation of the HAT. MYST and p300/CBP family HATs contain a cysteine-rich, zinc-binding domain, which could facilitate binding directly to DNA. Some MYST family HATs contain in addition an N-terminal chromodomain, which binds to methylated lysine residues. HAT = catalytic histonae acetyltransferase domain, BRO = bromodomain, CHR = chromodomain, ZN = zinc-binding domain, KIX = kinase-inducible domain.

HAT function

Although most HATs are nuclear enzymes (A-type) which acetylate histones that are attached to the DNA, B-type HATs (KAT1, HAT4) are found in the cytoplasm as well as in the nucleus. B-type HATs modify free histones in the cytoplasm just after their synthesis, upon which they are transported to the nucleus and integrated in newly synthesized DNA, creating the nucleosome (11,12). Immediately after the histones are integrated in the DNA, they are deacetylated by HDACs and later reacetylated by nuclear HATs with new acetylation patterns (13-15). Nuclear HATs are transcriptional regulators involved in a variety of cellular processes like cell cycle control, differentiation, DNA damage repair and apoptosis. They operate in protein complexes, which increase the specificity of HATs to histone acetylation and target genes as well as expand the range of targets to non-histone proteins (Table 1). The different nuclear HAT subtypes, their function and protein complexes will be discussed below.

The GNAT family

GNAT family HATs (KAT2A, GCN5 and KAT2B, PCAF (p300/CBP-associated factor)) acetylate histone H3 and histone H4. Both KAT2A and 2B are components of the human STAGA (SPT3-TAF9-GCN5 acetyltransferase) and ATAC (Ada Two-A containing) complexes, which are a large complex consisting of more than 20 components. The exact extent of functions of these complexes is still unknown, but involvement in chromatin structure modification and regulation of transcription as well as DNA damage repair have been identified (16). Although these two HATs have a sequence identity of 70% and seem to occupy the same protein complexes in humans, a large difference was shown in generating knockout mice. KAT2A mice turned out not to be viable, whereas KAT2B knockout mice were both viable and fertile, showing a remarkable difference in the function of the two enzymes (17).

Apart from their role in chromatin modification, several non-histone proteins have been identified as targets for KAT2A and 2B. KAT2A interacts with c-Myc, a transcription factor involved in transcription of proto-oncogenes. The c-Myc cofactor TRRAP (transformation/ transcription domain-associated protein) recruits KAT2A to c-MYC, which activates transcriptional activity (18). KAT2A is not the only HAT that acetylates c-Myc. Also KAT2B and KAT5 have been identified as acetyltransferases for this transcription factor, which will be further discussed in the "HATs in disease, cancer" section (21). In contrast, KAT2B acetylates p53, activating its transcriptional activity, and is therefore also associated with tumor suppression (22). KAT2B acetylates the enzyme ATP-citrate synthase (ACLY), which is involved in the citric acid cycle and synthesizes acetyl coenzyme A. Acetylation stabilized ATP synthase, whereas deacetylation by SIRT2 (NAD-dependent protein deacetylase sirtuin-2)destabilized the enzyme (19). This may function as a positive feedback mechanism, since acetyl coenzyme A is the cofactor and acetyl donor for all HATs. KAT2B also acetylates a nuclear hormone receptor, the androgen receptor (AR), which is coactivated by several HATs of different families, including KAT5, KAT7 and p300 (20,27).

The MYST family

The MYST family is currently the biggest family of human HATs. MYST family members show a high sequence similarity of 36-61%, but have a variety of different functions.

KAT5 (TIP60) is a global acetyltransferase of histone H4 and histone H2A and is involved in cell cycle progression and DNA damage repair (38). KAT5 is part of the NuA4 complex that contains 13 associated proteins in yeast of which 12 are conserved in humans. Among the proteins in this complex are inhibitor of growth 1 and 3 (ING1, ING3), which regulate cell cycle progression, apoptosis and DNA repair. Several non-histone targets for KAT5 have been currently identified. Ataxia telangiectasia mutant (ATM) is a protein kinase, which regulates DNA damage response in cells by phosphorylation of cell cycle check-point proteins and proteins involved in DNA damage repair. KAT5 was

Table 1: Human histone acetyltransferases: families, subtypes, complexes and substrates.

Family	Subtype	histone substrate	non-histone substrate	complex
cytoplasmic	KAT1 (HAT1)	H4K5, H4K12, H2AK5		
GNAT	KAT2A (GCN5)	H3K14, H4K8, H4	c-MYC (18)	ATAC, STAGA
	KAT2B (PCAF)	H3, H4	ACLY (19), AR (20), c-MYC (21) , p53 (22)	ATAC. STAGA
MYST	KAT5 (TIP60)	H4, H2A	ATM (23), NR1D2 (24), AR (20), c-MYC (21)	NuA4
	KAT6A (MYST3, MOZ)	H3	p53 (25)	MOZ/MORF
	KAT6B (MYST4, MORF)	H3	CBP (26)	MOZ/MORF
	KAT7 (MYST2, HBO1)	H4	AR (27)	HBO1
	KAT8 (MYST1, MOF)	H4K16	p53 (28)	MSL/MSL1v1
p300/CBP	p300	H2, H3, H4	CART1 (29), SIRT2 (30), PCNA (31), AR (20), p65 (32), p50 (33)	
	CBP	(H2), H3, H4	FOXO1 (34), PCNA (31), β-catenin (35-37), p53 (22)	

shown to acetylate ATM following DNA damage, which was essential for its function (23). Additionally, KAT5 acetylates the orphan nuclear hormone receptor NR1D2, which modulates apolipoprotein C-III gene expression (24).

Two remarkable members of the MYST family are KAT6A and 6B (MYST3, MOZ and MYST4, MORF). KAT6A and 6B are not found in drosophila or yeast: They are vertebrate specific. They function as part of a complex with inhibitor of growth 5 (ING5), Esa1-associated factor 6 orthologue (EAF6), and the bromodomain-PHD finger protein (BRPF) 1, -2, or -3 (39). This complex has mainly H3 acetyltransferase activity. KAT6B acetylates p53 at K120 and K382 and enhances its tumor suppressor function (25). KAT6A has been shown to interact with CBP, a HAT from the p300/CBP family, thereby inhibiting its function (26). This suggests that this HAT might not only regulate gene transcription and signaling transduction, but influences other HATs as well. Both KAT6A and 6B have been associated with embryogenesis and the maintenance of healthy stem cells. KAT6A has been shown to be essential for the maintenance of neuronal and hematopoietic stem cell proliferation capacity through inhibition of the cyclin-dependent kinase inhibitor p16INK4a and interaction with the BRPF proteins (40-42). KAT6B is the homologue of a mice histone acetyltransferase called “querkopf” (translated “squarehead”). Querkopf is called after the craniofacial abnormalities that occur in querkopf mutant mice. Studies in these mice revealed that this HAT is essential for the development of the central nervous system in the cerebral cortex. It was suggested that querkopf regulates the differentiation of neuronal cells (43). Additionally, both KAT6A and 6B were shown to interact with Runx2, a transcription factor involved in differentiation of osteoblasts and formation of the skeleton (44). They are also known to form fusion proteins often found in different types of leukemia, which will be discussed further in the section “HATs in disease”.

The majority of the bulk H4 acetylation is done by KAT7 (MYST2, HBO1). KAT7 was initially discovered as Histone acetyltransferase Binding to ORC (origin recognition complex) 1 (HBO1) in yeast and Drosophila. ORC is a protein involved in initiation of DNA replication and the interaction with HBO1/KAT7 is conserved in humans (45). KAT7 is part of the HBO complex which, as KAT6A and 6B complexes, contains ING5 and BRPF2. This suggests a role of KAT7 in cell cycle progression and cell growth and erythropoiesis (46,47). KAT7 also interacts with a nuclear receptor, the androgen receptor, and modulates its function (27). KAT8 (MYST1, MOF (males absent on the first)) is part of the male-specific lethal (MSL) complex. In Drosophila, the MSL complex is crucial for dosage compensation in male flies. The complex is evolutionary conserved in mammals and a human homologue has been described. In humans, the MSL complex is responsible for acetylation of specifically histone H4 lysine 16 (H4K16) and plays a role in gene transcription and cell cycle progression (48). KAT8 is also part of a second complex, KAT8-MSL1v1, which acetylates K120 of p53, a tumor suppressor protein. This complex contains WDR5, a part of the mixed lineage leukemia (MLL) complex, which is associated with acute leukemia (28). KAT8 knockout mice were not viable, showing abnormal chromatin architecture and lack of H4K16 acetylation. This points out the importance of this HAT in eukaryotic embryogenesis (49). Indeed, it was shown that KAT8 complexes were essential for the differentiation and development of mouse embryonic stem cells, both by regulating

cellular homeostasis and proliferation as well as histone acetylation (50). Also in human embryonic cells, KAT8 was essential for self-renewal and pluripotency (51).

The p300/CBP family

The p300/CBP HAT family, consisting of p300 and CBP (cAMP response element binding protein (CREB) binding protein), is the most studied among HAT families. HATs from this family acetylate all four core histones and several non-histone proteins. p300 and CBP both bind and activate phosphorylated CREB protein, which is involved in cAMP gene regulation (52). Knockout mice of either p300 or CBP died early in embryogenesis due to defective blood vessel formation. Even double heterozygous mice were not viable, showing the importance of these enzymes (53). p300 and CBP acetylate several non-histone proteins. The first interaction of p300 and CBP with a non-histone protein discovered, was with the tumor suppressor protein p53. CBP and p300, like KAT2B, acetylate p53 and thereby induce a conformational change that activates the transcriptional activity of p53 (22). After this, many more interaction partners were discovered. For example, they acetylate proliferating cell nuclear antigen protein (PCNA), which leads to removal and degradation after nucleotide excision repair. This degradation is important for the re-stabilization of the DNA (31). p300 has been shown to acetylate cartilage homeoprotein 1 (CART1), a protein of which the exact function is still unknown, but that might be involved in chondrocyte and cervix development. CBP co-activates CART1 in the presence of p300 (29). p300 also inactivates deacetylases by acetylation, for example SIRT2, which is known to deacetylate histones and inactivate HATs (including p300) by deacetylating them (30). This leads to a positive feedback, increasing acetyltransferase activity and histone acetylation. Both p300 and CBP have been shown to acetylate β-catenin, a component of the Wnt signaling pathway which is important in inflammation and cell proliferation. However, the influence of both HATs on β-catenin is strikingly different. Acetylation by CBP was suggested to regulate activity of β-catenin, inhibiting its role in cancer progression (36), whereas acetylation by p300, was shown to activate β-catenin and promote cancer (54,55). CBP was shown to also activate β-catenin, but this was independent of its acetyltransferase activity. This interaction was shown to be important for the maintenance of stem cell pluripotency (37). Having noticed this difference between p300 and CBP, Teo and Kahn (56) did an interesting proposal for a model in which the cell, upon a certain stimulus, chooses between β-catenin interacting with p300 or with CBP, leading to either proliferation or differentiation. When β-catenin associates with CBP, the cell will proliferate and maintain pluripotency, but when β-catenin associates with p300, the cell will differentiate to a specific cell type.

CBP acetylates Forkhead box, class O 1 (FOXO1), which is involved in metabolic homeostasis in response to oxidative stress. Acetylation of CBP attenuates the transcriptional activity of FOXO1 by disrupting the interaction with the target DNA. On the other hand, association of CBP with FOXO1 was necessary for transcriptional activity, again showing the complexity of HAT function (57). Another Forkhead box protein, FOXP3, also interacts with p300. p300 can acetylate FOXP3, which controls FOXP3 levels in T cells and regulates the function of regulatory T cells. It has been suggested that

the influence of p300 on FOXP3 is cooperative with KAT5 and that KAT5 and p300 work together in regulating FOXP3 (58,59).

The variety of targets for HATs and the elaborate complexes they function in, result in a role for HATs in many cellular processes. The very details of these functions are still unknown, but in the last decade, increasing interest in these important epigenetic enzymes have resulted in a growing number of studies into their structure and function. This growing number of studies also resulted in an increased interest in the role of these HATs in disease state. It is therefore important to evaluate what opportunities there are in medicine for these enzymes and whether they could be new targets to meet medical need.

HATs in disease

Due to their influence on many processes, dysfunction of HATs has been associated with many diseases ranging from neurodegenerative diseases to cancer. This section will give an overview of what is currently known about HATs in cancer, inflammatory diseases, viral infections and neurological disorders (Figure 3).

Cancer

Cancer is the collective term for a broad group of diseases marked by tumor growth and metastasis of malignant cells. Some studies suggest that HATs act protectively against cancer. In tumor cells, HAT protein and global acetylation levels are changed and can be correlated with clinical outcome. For example, high levels of acetylated H3K9 and H4K16 were correlated with improved prognosis and patient survival in non-small cell lung cancer patients (60). A genetic factor was found in tumor cells of both mice and humans, where mutation or loss of the second allele of p300/CBP HATs was found in primary tumors of colorectal and breast cancer and in different cancer cell lines. It was suggested that the p300 gene could function as tumor suppressor gene (61). However, high p300 mRNA levels in colorectal cancer have been associated with poor prognosis whereas high expression levels of CBP predicted long-term survival (62). This shows that global acetylation or protein levels do not provide a clear answer to whether HATs are pro- or anti- cancer.

HATs can activate tumor suppressor proteins by indirect or direct acetylation. For example, p300 was shown to inactivate SIRT2, an HDAC, leading to increased p53 activity, which is a marker for tumor suppression. Direct acetylation of the tumor suppressor protein FOXO1 by CBP was shown to inhibit pancreatic tumor cell growth (34). This, however is in contrast to the finding that FOXO1 acetylation by CBP inhibited transcriptional activity (see “the p300/CBP family”).

On the contrary, there is increasing evidence that HATs can play a role in the onset and progression of cancer. Global histone acetylation has not only been correlated with improved prognosis, but also with tumor recurrence and negative clinical outcome.

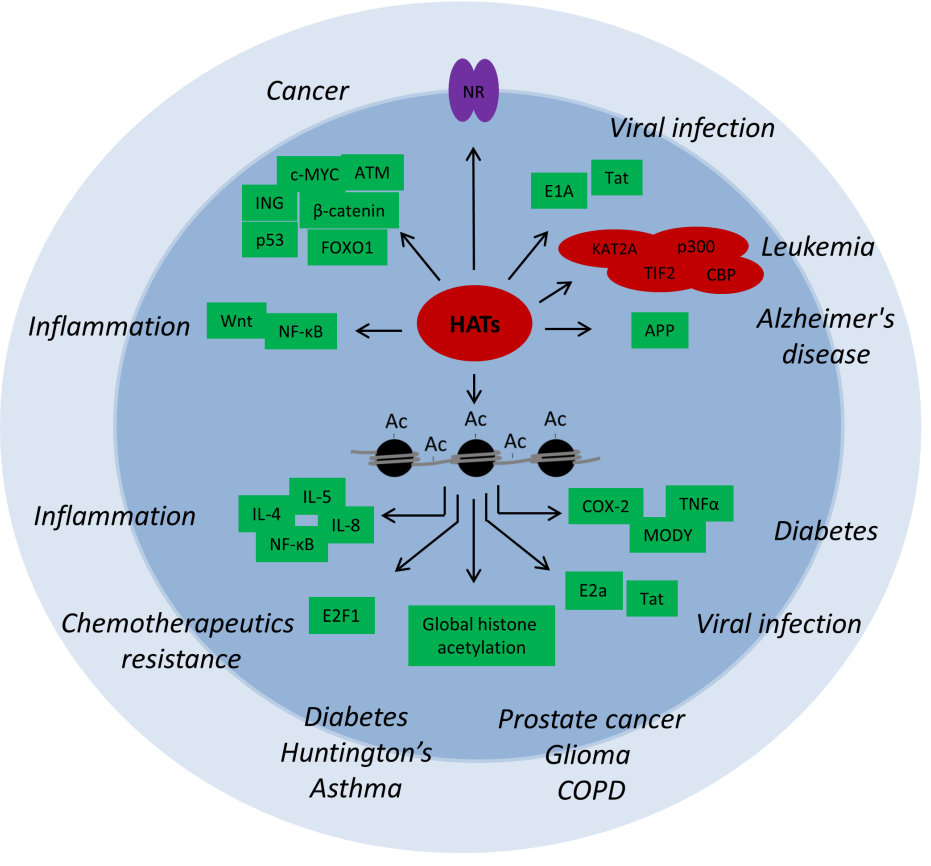

Figure 3: HATs are involved in several diseases including cancer, inflammatory diseases, viral infections and neuronal disorders. HATs exert their effects both through histone as well as non-histone substrate acetylation. Interaction with APP (amyloid precursor protein) is associated with progression of Alzheimer's disease; acetylation of c-MYC, ATM (ataxia telangiectasia mutant), ING (inhibitor of growth) proteins, β-catenin, p53, FOXO1 (forkhead box, class O 1) and nuclear receptors (NR) has been associated with cancer; Acetylation of the viral transcription factors E1A (adenovirus early region 1A) and Tat (trans-activator of transcription) promotes virus invasion. Fusion proteins of several HATs have been associated with acute leukemias. Changes in global histone acetylation levels have been found in diabetes, Huntington's disease, asthma, prostate cancer, glioma and COPD (chronic obstructive pulmonary disease). Acetylation of the promotors of different interleukins (IL) and NF-κB (nuclear factor kappa B) is involved in inflammation, of E2F1 (E2F transcription factor 1) in resistance to chemotherapeutic agents, of COX-2 (cyclooxygenase-2), MODY (maturity –onset diabetes of the young) and TNFα (tumor necrosis factor alpha) in diabetes, of E2a and Tat in viral infections.

Histone H3 and H4 of hepatic stellate cells were hyperacetylated in hepatocellular carcinoma (63) and in prostate cancer, acetylation of H3K18 was correlated with an increased risk of tumor recurrence (64). Lower levels of acetylated H3K18 predicted better survival of glioma patients (65). It is striking to see that acetylation of the same histone tail can predict opposite outcomes in different types of cancer. This suggests that the role of global histone acetylation depends on the tissue type and may be influenced by other, currently unknown factors.

Several HAT enzymes have been linked to different cancer types through their non-histone interaction partners. High levels of p300 predicted large tumor size and aggressive progression in patients with prostate cancer (66). A possible mechanism of this promotion of prostate cancer cell growth is acetylation of the androgen receptor by p300 and several other HATs (KAT2B, KAT5, KAT7) (67). The oncogene c-MYC is acetylated by KAT2A, 2B and 5, which slows its degradation, promoting cancer progression (21). In contrast, KAT5, as well as KAT6A, was shown to counteract c-MYC induced lymphomagenesis in in pre- or early- tumoral stage (68,69), showing that the same HAT can have opposite effects on oncogenes in different situations. Another example is the influence of CBP on β-catenin that was discussed under "HAT function-p300/CBP family".

Genetic rearrangements of HAT genes can lead to fusion proteins that are involved in acute leukemias. Fusion proteins like KAT6A-CBP, KAT6A-p300, KAT6B-CBP have different chromatin organizing functions than the singular HATs and were correlated with acute myeloid leukemia and benign uterine leiomyomata. The KAT6A-TIF2 fusion protein has been associated with self-renewal of leukemic stem cells by inhibiting CBP and transcription through p53 and nuclear receptors (26,70). Some HATs have been associated with tumor resistance against chemotherapeutics. KAT2B (GNAT family) and KAT5 (MYST family) have been shown to be overexpressed in cisplatin-resistant tumor cells (71,72). The protein kinase ATM was shown to be an important factor for cisplatin resistance in squamous cell carcinoma and acetylation by KAT5 is essential for its function, providing a possible pathway for KAT5 mediated cisplatin resistance in this type of tumor cells (73). KAT2B was also overexpressed in cells resistant to other chemotherapeutic agents and led to reduced apoptosis through enhancement of E2F1 expression, a transcription factor associated with different cancer types (72).

HATs have important functions in cell cycle progression, cell proliferation and differentiation as well as activation or inactivation of non-histone proteins like androgen receptors, oncogenes, tumor suppressor genes and HDACs. The pro- or anticancer action of HATs may depend on the balance between acetylation and deacetylation, on the type of tumor cells and the coaction of the HAT protein complexes.

Inflammatory diseases

HATs are involved in inflammation via several different pathways. HATs can regulate gene transcription of interleukins (IL), which are important mediators of inflammation. For example, CBP and p300 were shown to activate IL-5 expression as well as IL-8 and IL-4 (74-76). As previously mentioned, HATs play a role in Wnt signaling through β-catenin, which is involved in cancer. Wnt signaling also plays an important role in inflammation. This role does not seem to be mediated through β-catenin, but via cross-regulation with nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (77), which is also an important transcription factor for interleukins and many other inflammatory proteins.

NF-κB is a transcription factor that plays a key role in regulating inflammatory responses to stimuli like stress, cytokines and bacterial or viral antigens. The active form consists

of a homo- or hetero dimer between two Rel homology domain containing proteins: RELA/p65, RELB, NFKB1/p105, NFKB1/p50, REL and NFκB2/p52. The most prevalent form is the p50/p65 dimer. NF-κB is present in an inactive form in almost all cell types and rapidly induces an inflammatory response upon activation. In resting cells, NF-κB is bound to IκB, an inhibitory protein of NF-κB. In case of an inflammatory reaction, inflammatory cytokines induce ubiquitination and degradation of IκB, which releases NF-κB. NF-κB translocates to the nucleus, where it upregulates inflammatory gene expression. Malfunction of NF-κB has been associated with inflammation related diseases as autoimmune disorders, chronic obstructive pulmonary diseases (COPD), neurodegenerative diseases and cancer (78).

Histone acetylation plays an important role in NF-κB activity. Presence of HATs and HDACs at the gene promotor influences the activity of NF-κB by acting as coactivators or repressors of NF-κB mediated gene transcription (79,80). NF-κB itself is acetylated on multiple positions by HATs, activating or deactivating transcriptional activity, which results in a fine-tuning of NF-κB activity by HATs (81). p300 and CBP have been shown to increase the binding affinity of NF-κB to the DNA and decrease affinity for the inhibitory protein IκB by acetylating lysines 218, 221 and 310 of p65 (32). Acetylation of the p50 subunit by p300 on lysine 431, 440 and 441 was shown to increase binding to the DNA, enhancing transcriptional activity of NF-κB (33). However, acetylation of p65 can also inhibit transcriptional activity of NF-κB. p300 and KAT2B were shown to acetylate lysine 122 and 123, which increased its affinity for IκB (82).

Other post-translational modifications such as phosphorylation, methylation and ubiquitination work together with acetylation to regulate NF-κB function. For example, ubiquitination competes for the same lysine residue as acetylation on the p65 subunit, having an opposite effect (83). Phosphorylation of serines 276 and 536 of p65 was shown to precede acetylation of lysine 310 by p300 and determine association with either p300/CBP or HDAC-1 (84,85). Acetylation of lysine 310 of p65 was shown to impair methylation of lysines 314 and 315, preventing ubiquitination and subsequent degradation of p65 (86). Thus, NF-κB is regulated by HATs both on the promotor level as well as on the transcription factor itself. This suggests that HATs could be a target to modulate inflammation.

Inflammatory lung diseases

Inflammatory lung diseases are characterized by an increase in inflammatory gene expression, which correlates with the severity of the disease state. HATs and HDACs are important regulators of inflammatory gene expression and their levels are altered in inflammatory lung diseases. In blood monocytes of patients with asthma, increased HAT activity was observed, although no increase in gene expression was shown (87). An inhibitor of airway inflammation was shown to decrease histone H4 acetylation and a potentiator of the HDAC Sirtuin 1, SRT1720, was shown to suppress inflammation in a mouse model for asthma (88). In asthmatic patients HAT activity is increased. Interestingly, glucocorticoids reduce HAT activity in asthmatic patients to levels seen in healthy patients

(89). In contrast, in COPD there is no increase in HAT activity, but a decrease in HDAC activity. Consequently, histone H4 acetylation was increased at the IL-8 promoter, which activated transcription of this interleukin (90). The same hyperacetylation of histone H4 at the IL-8 promotor was seen in cystic fibrosis (91). Not only the NF-κB pathway but also Wnt signaling, another very important inflammatory pathway, is affected by HAT activity. β-catenin is the main mediator of this pathway by enhancing transcription of inflammatory genes. As discussed in section "HAT function-p300/CBP family", the HATs p300 and CBP, have an important influence on this factor. In pulmonary fibrosis, it was shown that inhibiting the CBP/β-catenin interaction attenuated and even reversed disease by influencing the Wnt signaling pathway (35).

Cardiovascular diseases

In many cardiovascular diseases like diabetes and atherosclerosis, inflammatory processes play a major role in pathogenesis. Histone acetylation of inflammatory gene promotors and HAT/HDAC activity have been shown to be dysregulated in cardiovascular diseases. Global histone acetylation and histone acetylation patterns have been shown to be important in proliferation of smooth muscle cells and matrix remodeling, which are important processes in atherosclerosis and restenosis (92). In diabetic type-2 patients, inflammation has been associated with increased insulin resistance. Both recruitment of NF-κB and direct acetylation of inflammatory gene promotors, like TNFα and COX-2 has been observed in diabetic patients (93). Acetylation of several non-inflammatory genes plays a direct role in diabetes. Regulation of insulin gene expression by glucose, an important factor that is dysregulated in diabetes, was shown to be mediated through hyperacetylation of histone H4 on the gene promotor (94). Histone acetylation has been shown to be important for the dysregulation of several MODY (maturity –onset diabetes of the young) genes, which are then responsible for a type 2-like diabetes that starts at a young age (95).

Other inflammatory diseases

HATs have been associated with a number of other inflammatory diseases. In patients with rheumatoid arthritis, treatment with TNFα inhibitors, HDAC inhibitors or rituximab changed levels and activity of HATs and HDACs (96). Through their influence on inflammatory pathways like NF-κB, HATs have been linked to acute pancreatitis (97), renal injury (98) and granulomatosis with polyangiitis (Wegener's granulomatosis) (99).

Viral infections

Several human HATs interact with the HIV-1 transactivator protein (Tat). Tat is a viral transcription factor that is essential for the transcription of viral DNA and additionally works as a toxin for host cells. Tat recruits p300 and CBP to the viral promotor site, where acetylation of histone H3 and H4 enables viral mRNA transcription (100,101). Tat itself is acetylated by p300 and KAT2A, which is necessary for its transcriptional activity (101-103). The MYST family HAT KAT5 was initially identified as 60 kD Tat interactive protein (Tip60) even before its HAT activity was discovered. Unlike with other HATs, binding of KAT5 and Tat inhibited KAT5 acetylation activity rather than promote Tat activity (104).

HIV-1 is not the only virus using the hosts HATs. The adenoviral oncoprotein E1A binds p300/CBP and KAT2A/B shortly after invading and consequently may interfere with cellular processes (105). Type 12 of the E1A protein complexes with p300 and CBP, which has transcriptional activity on the viral E2a promoter. The E2a protein is essential for virus replication (106). Taken together, it seems that viruses like HIV-1 and adenovirus recruit HATs for efficient invasion of the host. HAT inhibitors may be suggested as potential antiviral therapeutics, although no extensive research has currently been done to develop HAT inhibitors for this purpose.

Neurological disorders

Rubinstein-Taybi syndrome is a syndrome characterized by growth impairment, mental retardation and aberrant morphologies like broad thumbs and halluces and distinct facial features. The syndrome is caused by mutations in the genes of the HATs CBP and, in fewer cases, p300. In Rubinstein-Taybi syndrome caused by mutations only in the p300 gene, physical symptoms are less severe and patients often have normal thumbs and halluces. The occurrence of both physical and mental symptoms caused by mutations in a single gene, suggests that these HATs are involved in development of the skeleton and in processes of learning and memory (107). Neurodegenerative disorders as Alzheimer's disease and Huntington's disease are complicated and progressive diseases, which include neuronal degradation and protein accumulation. HATs may play a role in onset and regression of these diseases through their function as transcriptional regulators. A change in global histone acetylation, particularly H3, was seen in cellular and animal models of Huntington's disease, suggesting a possible role for HATs and HDACs in this neurodegenerative disease (108). KAT5 was shown to interact with the neuronal protein amyloid precursor protein (APP), which is the main protein involved in the onset of Alzheimer's disease. APP is cleaved in an intracellular and extracellular part. The extracellular part forms β-amyloid fibers, which are the main factors in Alzheimer's disease. The intracellular part associates with KAT5 and plays a role in gene transcription, possibly contributing to the disease state (109-111). Although little is known about their exact role, HATs appear to be involved in neurodegenerative diseases through regulation of transcription and association with disease proteins. Whether they will become a target in these diseases remains to be investigated.

HAT inhibitors

HATs have many different functions, different subtypes can even have contradictory functions and there seems to be a delicate balance between HATs and their interaction partners. Not only in their physiological function, but also in pathological states, HATs have many different roles and the details of their involvement are yet unknown. The development of therapeutics targeting HATs is therefore still in a very early stage. The current focus of research is to develop HAT modulators, in this case also referred to as chemical probes, that can be used as tools to study the HAT enzymes and form a basis for potential therapeutics. With inhibitors or activators, the activity of enzymes can be modified, mechanisms of action can be elucidated and difficulties that occur with generating knockout mice can be avoided. Inhibitors can additionally be used to

study the potential for HATs as a molecular target in disease. However, before newly developed inhibitors or probes are taken further into clinical trial research, they must be considered promising by one of the larger pharmaceutical companies. However, there is no concluding set of criteria to define a chemical compound as "promising". There have been some suggestions from the scientific community for requirements that must be met by a certain chemical compound to be seen as useful. The Structural Genomics Consortium (SGC), for example, hold its own set of requirements for the probes that are developed within the consortium. A good chemical probe is a compound that I) has in-vitro potency (IC_{50} or K_d) of less than 100 nM, II) has more than 30-fold selectivity over other subfamilies and III) shows on-target effect in cells at less than 1 µM (http://www.thesgc.org/chemical-probes). However, the potency needed to have effect in-vivo, differs for different targets, making this an easy-to-follow rule of thumb, but giving no real guaranty for success. A broader set of guidelines is given by Steven Frye (112), which essentially covers the same issues as the SGC criteria:

1. *Molecular profiling:* A quality chemical probe has sufficient in vitro potency and selectivity data to confidently associate its in vitro profile to its cellular or in vivo profile.
2. *Mechanism of action:* A quality chemical probe has sufficient mechanistic data versus its intended molecular target to enable interpretation of its qualitative and quantitative effect (dose dependency) on a target-dependent action in either a cell-based assay or a cell-free assay that recapitulates a physiologic function of the target.
3. *Identity of the active species:* A quality chemical probe has sufficient chemical and physical property data to permit utilization in in vitro and cell-based assays with interpretations of results attributed to its intact structure or a well-characterized derivative.
4. *Proven utility as a probe:* A quality chemical probe has sufficient cellular activity data to confidently address at least one hypothesis about the role of the molecular target in a cell's response to its environment.
5. *Availability:* A quality chemical probe is readily available to the academic community with no restrictions on use.

But, as discussed by Thomas Kodadek (113), the criteria for chemical probes are different from the criteria for new drugs. Probes must be selective for a target and applicable in research. Drugs should mainly have the desired effect on the pathological condition, a lack of side effects and be sellable to patients. Significant effort has been made to develop HAT inhibitors or probes and studies show promising results for HAT inhibitors to become therapeutics to meet medical need. This section will discuss the current state of the art concerning HAT inhibitors (Figure 4).

Bisubstrate inhibitors

One class of HAT inhibitors are the bisubstrate inhibitors. Bisubstrate HAT inhibitors are synthetic molecules that consist of coenzyme A attached to a peptide that resembles part of the histone tail connected by a linker. The shortest bisubstrate inhibitor is Lysine-Coenzyme A (Lys-CoA), which is non-selective between HATs, however, selective

bisubstrate inhibitors have been made by modifying the peptide part to fit specific subtypes. Several bisubstrate inhibitors have been shown to inhibit transcriptional activation for both p300 and KAT2B (114). For KAT5, more potent multivalent peptide inhibitors were developed by methylating the peptide segment. This revealed interesting results on the influence of the KAT5 chromodomain (115). However, due to their chemical properties these inhibitors suffer from limited cell/permeability and metabolic instability. This is a major drawback when doing cell-based assays and limits the potential therapeutic possibilities of these inhibitors.

Natural products and derivatives

Plant derived natural products have been investigated for HAT modulation and this has resulted in the first class of small-molecule inhibitors for the different families of HATs. Garcinol is one of the major active components found in kokum, a spice extracted from the fruit of the Garcinia indica tree. It is a potent inhibitor of both KAT2B and p300, which has been investigated for its anti-cancer activity. Human cancer cell lines were more sensitive to irradiation after treatment with garcinol due to inhibition of non-homologous end joining (116). In a mouse-model of colon carcinogenesis, treatment with garcinol decreased tumor size and incidence. Additionally, a reduction of inflammatory mediators was observed (117). Based on garcinol, several specific and non-specific derivatives have been developed that showed both inhibitory as well as activating properties (118-120). One example is a recently published noncompetitive p300/CBP benzylidenebarbituric acid that was reported to be cell-permeable and selective for the p300/CBP family over KAT2B (121). Curcumin is a widely investigated natural product in medicine that is derived from turmeric, a spice used in Chinese and Indian traditional medicine (122). Among HATs, it inhibits p300/CBP and is suggested to be an allosteric inhibitor that does not bind to the binding sites of either histone tail or acetyl coenzyme A. Curcumin has many potential clinical applications and is currently investigated in about 120 clinical trials for diseases ranging from cancer to Alzheimer's disease, alone or in combination with other drugs (123). Nevertheless, from the structure of curcumin, it is clear that curcumin is a potentially reactive molecule. It has a phenolic structure that can function as an anti-oxidant and the unsaturated ketone can participate in a michael addition reaction with anions of alcohols and thiols, for example on proteins (124). Due to its chemical properties, it is likely that the biological effect of curcumin is not only due to its inhibiting effect on HATs, but also originates from its chemical reactivity with other proteins as well as reactive oxygen species.

Plumbagin is another non-competitive p300 inhibitor, which has been reported to induce apoptosis and cell-cycle arrest in human lung cancer cells (125,126). Quercetin is a well-known and intensively studied natural product of the flavonoid group of plant metabolites. It is most studied for its protective effects against cancer (127) and has been shown to reduce HAT activity, probably modulating p300/CBP activity (128). Gallic acid is found abundantly in many plant species, for example gallnuts, tea and several berries. It is a non-selective HAT inhibitor and has also been reported to inhibit carbonic anhydrase (129). Gallic acid has been linked to inhibition of NF-κB acetylation and suggested as a possible treatment for Alzheimer's disease and lung cancer (130,131).

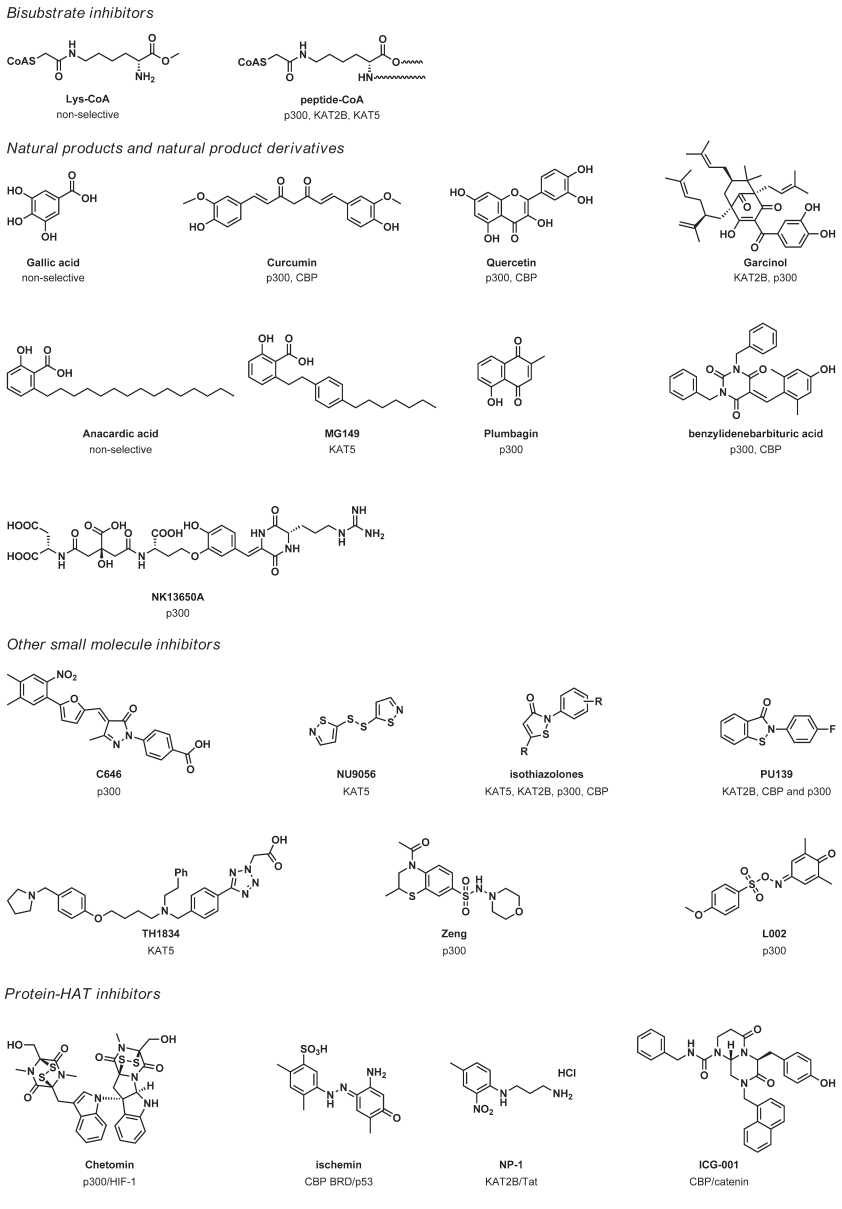

Figure 4: HAT inhibitors and their reported HAT targets. Bisubstrate inhibitors: bisubstrate HAT inhibitors are synthetic molecules that consist of CoA attached to a peptide that resembles part of the histone tail connected by a linker. These inhibitors are selective between different HAT subtypes. However, due to their chemical properties these inhibitors suffer from limited cell/permeability and metabolic instability. Natural products: natural product inhibitors of HATs are generally non-selective and inhibit a range of targets besides HATs in addition to anti-oxidant activity. Natural product derivatives: derivatives of natural products have been developed that are selective between HAT families. Other small molecule inhibitors: structure-based design and high throughput screening have resulted in new, structurally unrelated small molecule inhibitors. Protein-HAT inhibitors: Inhibiting bromodomains or the interaction between HATs and other proteins, is a promising way of targeting specific HAT pathways.

Anacardic acid is a natural product derived from cashew nut shell liquid, a byproduct in cashew nut processing. Like other natural product inhibitors of HATs, anacardic acid has been shown to sensitize human tumor cells to the cytotoxic effects of ionizing radiation (132). It has been reported to inhibit a diverse number of enzymes in addition to HATs (133-137). In fact, derivatives of anacardic acid have been described to be active on enzymes from all different families of HATs. For p300 it was shown that the amide derivative N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide was selective for p300 over KAT2B and enhances the p300 HAT-dependent transcription from in vitro assembled chromatin template (138). Other derivatives reduced p300 mediated acetylation in MCF7 breast carcinoma cells (139). For KAT2B, a derivative showed improved inhibitory potency compared to anacardic acid and inhibited histone acetylation in HEP G2 cells (140). Interestingly, an analogue of anacardic acid, pentadecylidenemalonate, was found to inhibit p300/CBP while activating KAT2B. It showed apoptotic effects in human leukemia U937 cell line (141). Also for KAT2A, anacardic acid showed inhibitory potency. It induced hypoacetylation of histone H3 in Plasmodium falciparum and blocked the growth of both chloroquine-sensitive and -resistant strains (142). In 2011, the anacardic acid derivative MG149 was reported to inhibit KAT5 (143). MG149 was shown to have an effect on a select set of nuclear processes including p53 and NF-κB gene expression (144). Not only plant-derived natural products can be HAT inhibitors. From a screen for microbial metabolites in a strain of penicillin, the natural product and peptide mimic NK13650A was discovered. This inhibitor was shown to be selective for p300 over KAT5 and inhibit androgen receptor activation as well as growth of prostate cancer cells (145).

One might appreciate that many of these natural products and derivatives are non-selective with a wide range of biological targets. They often have phenolic structures and therefore have antioxidant properties. This can possibly give additional biological effects separate from the on-target effects. For the development of drugs, for example against cancer, this might not be a problem, but in case of probes selectivity is an important issue. Therefore, other methods have been used to find starting point for the development of HAT modulators.

Other small molecule inhibitors

In addition to bisubstrate inhibitors and natural products, a series of small-molecules have been developed using a variety of methods. High throughput screening of databases consisting of a large number of synthetic compounds is a common method to look for new inhibitors with new scaffolds. For such a screen, an efficient assay to test the compounds is needed and efforts have been done to develop assays to screen for inhibition of HAT activity (146). From a high throughput screening, the class of isothiazolones was found to inhibit the HAT subtype KAT2B. Several derivatives were made that inhibited KAT2B and p300. These isothiazolones were shown to inhibit cell growth and proliferation of human cancer cell lines (147,148). The isothiazole NU9056 has been shown to inhibit KAT5 and has been suggested as application for treatment of prostate cancer. NU9056 induced apoptosis in prostate cancer cell lines via activation of caspase 3 and caspase 9 and was correlated with the physiological function of KAT5 by effecting the androgen receptor

and ATM (see also section “HAT function”) (149). The pyridoisothiazolone PU139 is a non-selective HAT inhibitor with activity on KAT2B, CBP and p300, which was shown to inhibit neuroblastoma cell growth in xenograft mice. Interestingly, this inhibitor was also found to control the transmission of schistosomiasis, a disease caused by the parasitic worm Schistosoma mansoni, by inhibiting egg formation (150,151). A high throughput screening in cancer cell lines yielded the p300 inhibitor L002, which showed activity in mouse xenograft models as well (152).

For p300, computational design has been used for the development of new inhibitors. Bowers et al. (2010) used virtual screening of a database of commercially available compounds to find C646, which is currently the most potent small molecule inhibitor of p300 (153). Zeng et al. (2013) used the same computational technique to discover an inhibitor 4-acetyl-2-methyl-N-morpholino-3,4-dihydro-2H-benzo[b][1, 4]thiazine-7-sulfonamide referred to as ‘Zeng’ in figure 4 (154).

With the availability of crystal structures of HATs and the identification of small molecule inhibitors, structure-based in-silico design has become a possible method for development of HAT inhibitors. Based on pentamidine (PNT), an inhibitor of endoexonuclease activity that was found to inhibit KAT5, TH1834 was designed. This inhibitor was shown to inhibit KAT5 selectively over KAT8 and increased apoptosis in breast cancer cells following radiation (155,156).

Protein-HAT inhibitors

Another way to inhibit HATs is to target their interactions with interaction partners. HATs interact with many other proteins in addition to histones and inhibition of these interactions might provide more specific inhibition in a biological context.

In a high throughput screening, ICG-001, an inhibitor of the interaction between CBP and β-catenin was discovered. This inhibitor was specific for the CBP/β-catenin interaction and did not inhibit the p300/β-catenin interaction. This is essential, since CBP and p300 have differential roles in their activation of β-catenin (see also “HAT function – p300/CBP family) (157). Another inhibitor of a HAT-protein interaction is chetomin, a metabolite from the fungal species Chaetomium. Chetomin inhibits the interaction between p300 and hypoxia-inducible factor-1 (HIF-1), a protein characteristically overexpressed in malignant gliomas, which is also a known interaction partner of β-catenin. Treatment of malignant glioma cell lines attenuated the hypoxia-induced radioresistance, suggesting that chetomin could possibly be used as a strategy to sensitize human malignant gliomas to radiotherapy (158).

The bromodomain inhibitors are a class of molecules that inhibit the interaction of the bromodomain with the acetylated lysine on histones or other proteins. There are several classes of proteins containing bromodomains, including HATs from the GNAT and p300/CBP family (see “Histone acetylation”, Figure 2B) (159). Bromodomain inhibitors are mainly known for their inhibition of the bromodomain and extra-C-terminal domain (BET) protein family and a few of these inhibitors are currently in clinical trials for treatment of NUT midline carcinoma and hematological malignancies (160). Inhibitors of

HAT bromodomains have been described for CBP and KAT2B. A small molecule inhibitor of the CBP BRDs is ischemin, an azobenzene-based inhibitor. Ischemin was shown to inhibit the interaction of CBP with p53 and efficiently prevent apoptosis in ischaemic cardiomyocytes (161), although the selectivity of ischemin for the CBP BRD has not been investigated yet. Also a collection of cyclic peptides were designed, based on the NMR structure of CBP, to inhibit CBP-p53 interaction inhibitor (162). In case of KAT2B, N1-aryl-propane-1,3-diamine derivatives, for example NP-1, were found as bromodomain inhibitors for the interaction with the acetylated HIV protein Tat and the inhibitors were shown to inhibit HIV-1 replication in cellular assays (163,164). Although HAT bromodomain inhibitors are still in early development, they may be developed towards a class of therapeutics for cancer or viral diseases in the coming years.

Therapeutic possibilities of HAT inhibitors

HATs form a diverse group of enzymes that play an important role in the post-translational modification of histones. They regulate gene transcription and contribute to the histone code, which is one of the main epigenetic mechanisms influencing signaling cascades. HATs function in diverse protein complexes that determine substrate specificity and expand the range of targets. HATs have been associated with several different diseases both through global and specific acetylation of histones and acetylation of non-histone substrates.

Both activity of HDACs and HATs have been associated with different types of cancer. This might seem contradictory, but although the direct action of HATs and HDACs is opposite, the substrates of these enzymes differ. Inhibiting acetylation may not necessarily mean deacetylation and vice versa. The pro- or anticancer action of HATs may therefore depend on the balance between acetylation and deacetylation, on the type of tumor cells and the co-action of the HAT protein complexes. HATs are associated with different inflammatory diseases including asthma, COPD, rheumatoid arthritis and several cardiovascular diseases. In asthma, an increase in HAT activity is seen and in COPD a decrease in HDAC activity. Also in other inflammatory diseases, acetylation seems to promote disease. This suggests that hyperacetylation or changes in the acetylation pattern are involved in inflammatory diseases and that patients will benefit from a restored balance between acetylation and deacetylation. HAT inhibitors can all cause a reduction of acetylation, but in the end this might lead to very different responses due to the many different functions of HATs. It is therefore important to investigate which HATs are involved in inflammatory diseases and which HATs need to be targeted to restore the balance.

HAT inhibitors may also have a therapeutic application in viral diseases. HATs promote viral replication and invasion of the host by interaction with viral proteins. The interaction of HATs with viral proteins like Tat or E1A, might be an interesting target for further exploration with inhibitors. Some evidence exists that correlates HAT activity with neurodegenerative diseases. HATs seem to play a role in transcriptional regulation in Alzheimer's and Huntington's disease, but further research must be done to confirm that this is a feasible target for HAT inhibitors.

Due to the large differences in function and similarities in structure of HAT subtypes, selectivity might become an important issue to prevent side effects. Already, inhibitors have been developed that are selective between HAT families, although selectivity between different subtypes within one family will, due to the structural similarity of the catalytic domains, be very challenging. Natural product inhibitors of HATs are generally non-selective and inhibit a range of targets besides HATs, in addition to anti-oxidant activity or protein reactivity. This might be useful when targeting multiple targets simultaneously, but can cause problems due to unwanted or unpredicted side-effects. Certainly as chemical probes, these inhibitors are not applicable. Some derivatives of these natural products have been developed that are selective between HAT families. Allosteric inhibitors have the potential to be selective between subtypes of the same family, since there is much variation in N- and C-terminal regions, but the catalytic domain is almost identical for all HATs. Structure-based design and high throughput screening have resulted in new, structurally unrelated small molecule inhibitors. These inhibitors have been shown to exhibit promising biological effects, although still limited to in-vitro studies. HATs interact with many proteins, modulating their activity where sometimes acetylation activity is not even required, for example through bromodomains or other protein interaction domains. Inhibition of these protein-protein interactions is a promising way of targeting specific HAT pathways.

Knowledge about HATs, HAT subtypes and the correlation with histone- and non-histone acetylation is too fragmented to predict the effect of HAT inhibitors in human disease state as yet. However, the current research on HAT inhibitors shows promising results for these important epigenetic enzymes to become drug targets for therapeutics against disease.

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